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The neonicotinoid pesticide, imidacloprid, affects *Bombus impatiens* (bumblebee) sonication behavior when consumed at doses below the LD50

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Abstract We investigated changes in sonication (or buzz-pollination) behavior of *Bombus impatiens* bumblebees, after consumption of the neonicotinoid pesticide, imidacloprid. We measured sonication frequency, sonication length, and flight (wing beat) frequency of marked bees collecting pollen from *Solanum lycopersicum* (tomato), and then randomly assigned bees to consume 0, 0.0515, 0.515, or 5.15 ng of imidacloprid. We recorded the number of bees in each treatment group that resumed sonication behavior after consuming imidacloprid, and re-measured sonication and flight behavior for these bees. We did not find evidence that consuming 0.0515 ng imidacloprid affected the sonication length, sonication frequency, or flight frequency for bees that sonicated after consuming imidacloprid; we were unable to test changes in these variables for bees that consumed 0.515 or 5.15 ng because we did not observe enough of these bees sonicating after treatment. We performed Cox proportional hazard regression to determine whether consuming imidacloprid affected the probability of engaging in further sonication behavior on *S. lycopersicum* and found that bumblebees who consumed 0.515 or 5.15 ng of imidacloprid were significantly less likely to sonicate after treatment than bees who consumed no imidacloprid. At the end of the experiment, we classified bees as dead or alive; our data suggest a trend of increasing mortality with higher doses of imidacloprid. Our results show that even modest doses of imidacloprid can significantly affect the likelihood of bumblebees engaging in sonication, a behavior critical for the pollination of a variety of crops and other plants.

Keywords Buzz pollination · Native bees · Pollination · *Solanum* · Tomato · Vibration

Introduction

Crops and wild plants depend on insects for pollination (McGregor 1976; Klein et al. 2007). Honeybees pollinate many crops, but wild bees are valuable pollinators for a significant number of crops globally (Klein et al. 2007). Because much of the human food supply depends on bees, concern has been expressed about recent population declines in honeybees (*Apis mellifera*) (Meffe 1998; van Engelsdorp et al. 2009) and wild bees (Gallai et al. 2009; Goulson et al. 2015). Pesticides may be one factor contributing to the wide-scale decline of pollinators (Potts et al. 2010), and many studies have investigated the effects of sub-lethal and chronic exposure on bees (Desneux et al. 2007; Gill et al. 2012; Henry et al. 2012; Whitehorn et al. 2012; Gill and Raine 2014).

One common pesticide used in the United States and much of the world is imidacloprid (Pollak and Vouillamoz 2011). This is a systemic and contact insecticide in the class of neonicotinoid (mimicking nicotine) pesticides (Mullins 1993; Tomizawa and Casida 2005; Gervais et al. 2010). It acts on acetylcholine receptors in the nervous system (Nagata et al. 1997) and has low mammalian toxicity (Mullins 1993), but causes paralysis and eventual death in insects (Kagabu et al. 2004). Imidacloprid is kills freshwater arthropods at concentrations of between several $\mu g\ L^{-1}$ to several thousand $\mu g\ L^{-1}$ (Beketov and Liess

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2008). Neonicotinoid pesticides have delayed effects that lead to death, but not within the time span of typical LD_{50} (lethal oral dose) measurements of 24 or 48 h (Beketov and Liess 2008; Rondeau et al. 2014). Concerns about the effects of neonicotinoids on pollinators led to an EU-wide ban on three neonicotinoid pesticides in 2013, including imidacloprid (Decourtye et al. 2013; European Commission 2013).

Neonicotinoids and their metabolites (also toxic to insects) have been reported in all tissues of plants, including pollen and nectar (Pohorecka et al. 2012). When applied to the soil, imidacloprid can be incorporated into nectar for up to ~230 days after application (Byrne et al. 2014). When imidacloprid is applied to seed coatings, the majority of the pesticide (up to 90 %) remains in the soil (Goulson 2013). The amount of pesticide found in pollen and nectar varies. The following values have been reported for imidacloprid residues: 10 ppb nectar and 14 ppb in pollen from squash (Stoner and Eitzer 2012), 16 ppb in buckwheat nectar (Krischik et al. 2007), and 12.8 ng mL^{-1} in nectar of citrus trees (Byrne et al. 2014). In the citrus experiment, Byrne et al. (2014) found that when imidacloprid was applied at the full label rate (1.02 L ha^{-1}), the highest reported value in nectar was 21.9 ng mL^{-1}; however, taking into account the total residues of imidacloprid and its toxic metabolites, the highest amount reported was 37.1 ng mL^{-1}. Furthermore, the highest amount of total residues in uncapped nectar from the hive comb of nearby honeybees was reported as 95.2 ng mL^{-1} (Byrne et al. 2014). Other researchers have estimated that honeybees foraging for nectar could consume between 1.1 and 4.3 ng of imidacloprid over their lives (Rortais et al. 2005).

The lethal oral dose (LD_{50}) of imidacloprid for bees has been measured in several studies, with significant variation. The LD_{50} for bumblebees has been reported at 40 ng per bee (24 h) and 20 ng per bee (72 h) (Marletto et al. 2003). For honeybees (*A. mellifera*), a more recent review highlights several studies that report the 48-h LD_{50} for honeybees to be in the range of about 4–104 ng bee^{-1} (Blacquiere et al. 2012).

Some studies report that imidacloprid irreversibly blocks nicotinic acetylcholine receptors in insects (Tennekes and Sánchez-Bayo 2011; Rondeau et al. 2014). Scarce data exists about whether a single, concentrated dose or a chronic, low dose of imidacloprid is more harmful to insects (Van den Brink et al. 2016). Different arthropods show different sensitivities to acute versus chronic doses (Roessink et al. 2013), and pesticides affect arthropods differently in summer versus winter (Van den Brink et al. 2016). Suchail et al. (2001) report that chronic exposure to imidacloprid was toxic to honeybees at a 60–6000 times lower dose than those required to produce the same acute effect.

Neonicotinoid pesticides are also known to have sub-lethal effects on bees. Learning ability in bumblebees was affected by even a small dose of imidacloprid (10 µl of 10 ppm imidacloprid in sugar solution) (Stanley et al. 2015a), and imidacloprid-treated pollen has been shown to reduce visual learning capacity in honeybees (Han et al. 2010). Asian honeybees (*Apis cerana*) showed reduced olfactory learning after consuming as little as 0.1 ng of imidacloprid (Tan et al. 2015). Furthermore, neonicotinoid pesticides have sub-lethal effects on bumblebees at the colony level—colonies consuming pollen and sugar water containing 6 and 0.7 mg kg^{-1} imidacloprid, respectively, had an 85 % reduction in production of new queens (Whitehorn et al. 2012).

Chronic, sub-lethal doses of neonicotinoids have also been shown to alter some aspects of pollination behavior in bees. Bumblebees experiencing chronic exposure to neonicotinoids change their pollination behavior by returning with less pollen (Feltham et al. 2014). Imidacloprid ingestion by honeybees has been shown to reduce the number of returning foragers and the number of foraging bouts per bee (Karahan et al. 2015). Other research suggests that pesticides impair the pollination services provided by bumblebees—bumblebee colonies that were exposed to field-realistic doses of pesticides for 13 days showed lower visitation rates to apple blossoms, which resulted in these apple trees producing fewer seeds than trees that were pollinated by untreated bees (Stanley et al. 2015b).

Experiments investigating the effects of chronic pesticide exposure are valuable in that they may mimic field-realistic experiences, but these types of experiments are typically unable to measure the amount of pesticide actually ingested by bees, because measuring the amount of contaminated nectar that a bee consumes in the field is difficult. The estimates for the amount of nectar a foraging bee consumes in a day vary widely. The consumption of sugar depends on the bee's energetic needs—Beutler (1951) suggests that honeybees consume 11 mg sugar per hour of flight, and Rortais et al. (2005) estimated that a honeybee foraging for nectar during the summer would consume 32–128.4 mg of sugar per day.

Bumblebee workers consume more nectar per day than honeybees. Furthermore, foragers consume sugar at a higher rate than nest workers (Rortais et al. 2005), likely due to the energetic demands of flying and collecting resources. Incubating *Bombus terrestris* queens metabolize approximately 600 mg of sugar per day, according to respiration rate data (Silvola 1984), and male bumblebees (*Bombus lucorum*) foraging for 4 h per day consume an average of 180 µl of 50 % sugar solution in 24 h—about 90 mg of sugar per day (Bertsch 1984). Laycock et al. (2012) reported individual bumblebees (*Bombus terrestris*)

consuming 400 mg of sugar syrup (1.27 kg L^{-1} fructose/glucose/saccharose) in a day.

All of these reports suggest that a *B. impatiens* worker, foraging for a full day, could reasonably consume 150—300 µl of nectar (50 % w/w sugar) per day. If that nectar had a field-realistic concentration of imidacloprid (10 µg L^{-1}) (Stoner and Eitzer 2012), then a forager could ingest 1.5–3 ng of imidacloprid during a day of foraging. This estimate is similar to Laycock et al. (2012), which reported *B. terrestris* individuals consuming 3 ng of imidacloprid in a day when they fed on sugar syrup with imidacloprid (10 µg L^{-1}).

Here, we investigate the effects of imidacloprid on an important aspect of bumblebee pollination behavior, upon which many crops and other plants depend—sonication, or buzz pollination. We examined bumblebees (*Bombus impatiens*) sonicating on the blossoms of tomato plants (*Solanum lycopersicum*); these flowers produce only pollen (no nectar), enclosed within tube-like anthers with small pores that release pollen when sonicated, or vibrated, by bees (Buchmann 1983; Thorp 2000). Bumblebees and other wild bees (but not honeybees) can produce these vibrations by contracting their flight muscles at high frequencies without flapping their wings.

We provided bees with doses of imidacloprid that were well below reported LD_{50} values in a single, concentrated treatment (Marletto et al. 2003 reported a 48-h LD_{50} of 20 ng per individual *B. terrestris*). Two of our treatment groups represent amounts of imidacloprid that a bee could reasonably ingest in a single day (0.0515 and 0.515 ng), and the highest treatment, represents an amount that a bee may consume over several days (5.15 ng). We quantified several aspects of sonication and flight behavior, including sonication frequency, sonication length, and flight frequency, before treatment. After treatment, we observed bees for up to several weeks, recording which bees resumed sonication behavior, and re-measuring sonication and flight variables in bees that did perform buzz pollination. Unlike past studies, which treated whole hives of bumblebees with chronic doses of imidacloprid (e.g. Gill and Raine 2014; Stanley et al. 2015b), we controlled the amount of imidacloprid ingested by individual bees by providing a single, measured dose in sucrose solution to each bee.

Materials and methods

Study organisms and foraging space

We purchased four class-A hives of *Bombus impatiens* from Biobest (http://www.biobestgroup.com). Two hives arrived on 10 Sept 2015, and another two hives arrived on 22 Sept 2015. Upon receiving the hives, we verified that queens were present in each, and we removed any males. Each hive was placed in a mesh cage that was 1.8 m long by 1.8 m tall by 0.6 m wide. These cages were placed in a pollinator-excluding greenhouse. The greenhouse had mesh walls and a plastic roof—thus the conditions inside the greenhouse were similar to outdoor conditions. We allowed bees to acclimate to the cages for at least 2 days prior to starting experiments.

The hives were enclosed in foam coolers for insulation, with small holes cut for entry and exit. Each cage contained a pollen feeder and a nectar feeder, providing pollen and nectar ad libitum. Nectar consisted of 342 g organic cane sugar per liter of water, or ~1 M sugar water. Pollen was purchased from Koppert Biological Systems (http://www.koppert.com) and ground with a mortar and pestle before placing ~2 g in a small, plastic dish. Pollen was replaced approximately every 3 days.

In addition to the artificial feeders, each cage contained a potted tomato plant (*Solanum lycopersicum*). We allowed bees to visit two varieties of cherry tomatoes with similar floral morphology, "Cherry Roma" and "Sweet 100 Hybrid". We used "Sweet 100 Hybrid" only on days when we did not have enough fresh "Cherry Roma" flowers. Each day that we observed the bees, we replaced the plant inside the bees' cage with a different plant that had been kept in a greenhouse that excluded pollinators. Thus, we were able to rotate tomato plants into the bee cages, constantly providing freshly-opened flowers for pollen foraging each day. We observed all four of the hives, typically between 10 a.m. and 4 p.m., until 16 Oct 2015. Because new individuals were treated daily, different numbers of individuals were observed for a range of dates.

We also recorded local weather data—pressure, temperature, relative humidity, and light intensity—at the time of each observation, using a weather station inside the greenhouse.

Marking individual foragers

We observed hives on each day with good weather (sunny and relatively still) until 16 Oct. When a bee was first observed foraging on *S. lycopersicum*, we captured her with an insect vacuum (2820GA, Bioquip, Rancho Dominguez, California) and transferred her from the aspirator tube into a queen-marking cage with plunger (The Bee Works, Oro-Medonte, Ontario, Canada). We gently pressed the bee against the mesh at the top of the tube to immobilize her while we marked her mesosoma.

We alternated marking the bees with paint or bee tags, to evaluate whether the marking method affects foraging behavior (Switzer and Combes, *in review, Journal of Melittology*). We marked about half of the bees with paint,

using oil-based paint pens (Sharpie, Oak Brook, Illinois), and the other half with bee tags—small, numbered plastic discs (~3 mm diameter; Queen marking kit, Abelo, Full Sutton, York, United Kingdom) attached to the mesosoma with superglue (Gel Control, Locktite, Henkel Corporation, Westlake, Ohio). After applying paint or affixing the tag with superglue, we used the outward vent from the insect vacuum to blow air onto the paint/glue for 30 s to dry it before releasing the bee back into the cage.

When we observed previously-marked individuals foraging for pollen on the *S. lycopersicum* plants, we reached into the cage with a shotgun microphone (SGM-1X, Azden, Tokyo, Japan) to collect an audio recording that included sonication and post-sonication flight sounds with a digital recorder (DR-100mkII, Tascam, Montebello, California). We then recaptured the bee for treatment with imidacloprid. We treated new bees with imidacloprid almost every day of the experiment and therefore did not have the same number of post-treatment observation days for each bee. This is accounted for by using Cox regression (below).

Imidacloprid treatments

After recapture, each marked bee was transferred to a clear, 1-L, plastic container with a vented lid, and held indoors without access to nectar or pollen for an average of 140 min. After being deprived of food, bees were randomly assigned to one of four treatment groups, and fed 10 µL of sugar water mixed with different amounts of imidacloprid (Pestanal, Sigma-Aldrich, St. Louis, Missouri), using a micropipette.

To prepare solutions for the treatment groups, we dissolved imidacloprid in deionized water and performed a series of dilutions to obtain the correct doses. Solutions were mixed and stored out of UV light, since imidacloprid breaks down quickly in water that is exposed to light at wavelengths between 200 and 300 nm (Zheng et al. 2003). We fed 10 µL of sugar solution mixed with imidacloprid to bees, resulting in the bees consuming 0.0515, 0.515, or 5.15 ng of imidacloprid. After feeding bees with 10 µL of imidacloprid solution, we used a clean micropipette to feed additional, untreated 1 M sugar water to the bees until they stopped drinking (stopped extending their proboscis). We deprived treated bees of food for at least 1 h after consuming the imidacloprid solution and additional nectar, and then released them back into the foraging cages.

The first time we observed a bee foraging on *S. lycopersicum* after treatment, we again recorded sonication and flight sounds with a microphone, then collected the bee and removed it from the experiment. The amount of time that elapsed between pre and post-treatment recordings was different for each bee—we account for the differences when we analyze the sounds, and we analyze the differences in time directly, using Cox regression (below).

At the end of the experiment (16 Oct 2015), we collected all of the remaining bees from the hives, recorded whether they were alive or dead, and used digital calipers to measure their intertegular (IT) span (the distance between the wing bases). We were unable to collect IT span measurements for all bees, as a small proportion of the paint marks or tags wore off during the course of the experiment—we marked a total of 212 bees during the experiment, and were unable to identify 17 of 100 marked with paint and 17 of 112 marked with tags. These individuals were dropped from our analysis.

Extracting data from audio

We used R (R Core Team 2015), with the packages seewave (Sueur et al. 2008) and tuneR (Ligges et al. 2013), to process the audio recordings and quantify sonication and flight sounds. We first listened to the recordings to identify the loudest, longest sonication sound, and recorded its length. We focused on the loudest, longest sonication, because we observed that bees often performed shorter, higher-frequency sonications on the petals of the flowers, and we wanted to exclude these from analysis. We classified sounds as a single sonication if there was no audible break for >0.2 s. After selecting the sequence for analysis, we used the "spec" function from the seewave package to calculate power spectral density, using a hanning window of 2048 points (Sueur et al. 2008). To determine the sonication frequency (the frequency at which the bee was vibrating), we selected the highest peak on the spectrum between 195 and 400 Hz (a reasonable range for sonication frequency).

To check the accuracy of the frequency obtained by this method, we generated a sine wave at the frequency identified as the highest peak, and compared it aurally to the audio recording of sonication. If the frequency returned from the spectrum was noticeably different from the audio recording (which can occur due to background noise), we used Audacity (Audacity 2015) to obtain the correct sonication frequency. Within Audacity, we selected the sonication portion of the audio recording, and plotted the spectrum (hanning window, 2048 points). We then generated sine waves at each of the frequencies corresponding to the peaks in the spectrum. We compared each of these sine waves to the recording, aurally, and chose the peak that corresponded most closely to the audio recording of the sonication.

We used the same process to quantify wingbeat frequency—selecting a portion of the recording that contained the bee flying, plotting a spectrum, and selecting the

highest peak. We used a range to 120–220 Hz for selecting the peak of wingbeat frequency, as wingbeat frequencies are typically lower than sonication frequencies (Switzer et al. 2016). We checked all wingbeat frequencies aurally in the same way as for sonication frequency.

Statistical analysis

To determine whether imidacloprid affected wingbeat frequency, sonication frequency, or length of sonication, we calculated the change in each behavior by subtracting post-treatment values from pre-treatment values. We observed a few bees sonicating on "Sweet 100 Hybrid" flowers (when "Cherry Roma" flowers were not available), and we initially excluded these individuals from our analysis of sonication and flight sounds, because we have previously found that bumblebees change sonication frequency and/or length on different species of plants (Switzer and Combes, *in review*, *Apidologie*). We reran the analysis, including the few bees that sonicated on *S. lycopersicum* "Sweet 100 Hybrid", and we found no differences in significance of coefficients associated with imidacloprid treatment; thus, these bees were included in the final analysis.

We performed multivariate multiple regression to determine if there were significant changes in the bees' behaviors—wingbeat frequency, sonication frequency, and sonication length. We were able to make comparisons only on bees that resumed sonication behavior after being treated with imidacloprid. Because very few bees in the 0.515 and 5.15 ng imidacloprid treatment groups were observed foraging for pollen on *S. lycopersicum* flowers after treatment, we dropped those two treatments from our analysis. Our initial model included imidacloprid treatment as the only dependent variable, and we used a series of likelihood ratio tests to determine if adding other covariates made the model significantly better. We chose this forward stepwise procedure—starting with a small model, and adding covariates—because we started with a small dataset and wanted to find variables that influenced the response variables while excluding variables that made small contributions (Armstrong and Hilton 2010). We suspected that environmental variables such as temperature might affect behaviors (Unwin and Corbet 1984), so we investigated the following weather covariates: temperature, atmospheric pressure, light intensity, and relative humidity. We also investigated mark type (paint or bee tag), intertegular span, the number of days between pre-treatment recording and post-treatment recording, and the hive number.

To evaluate whether imidacloprid treatment affects the probability that bees would resume sonication behavior, we used survival analysis techniques from the R package, survival (Therneau and Grambsch 2000). The data recorded includes the amount of time since diagnosis/treatment and whether or not an event occurs. The data are right-censored. For example, survival analysis has been used to examine the amount of time until seeds germinate (Manso et al. 2013). Here we used "collecting pollen from *S. lycopersicum* after being treated with imidacloprid" as our event. Our data are right-censored because some of the bees died, and others stayed alive but were never observed on *S. lycopersicum* after treatment, within the time limits of the study.

We used Cox proportional hazards regression to determine if there was a significant difference in the probability of bees resuming sonication behavior among the treatment groups. The coefficients from the Cox model can be used to estimate hazard ratios—the chance of the event occurring in the treatment group, relative to the control group. For example, in the highest treatment group, the coefficient is -2.73; $\exp(-2.73) = 0.065$, meaning that for a fixed point in time, individuals in the highest treatment group are about 0.065 times as likely to sonicate as bees in the control group.

We used Cox regression so we could include intertegular span and hive number as covariates. We centered the intertegular span variable before modeling to make interpretation easier. We also suspected an interaction between mark type and intertegular span, and between treatment and intertegular span, so we included interaction terms: intertegular span * mark type + intertegular span * treatment. We started with a full model (all covariates) and stepped backward, using likelihood ratio tests to determine if each covariate improved the model. We chose a backward stepwise procedure—starting with a large model and removing terms—because we had a priori reasons to include the interaction terms, and a forward procedure would not investigate interactions when the main effects are not significant. We used the R packages, ggplot2 (Wickham 2009) and ggfortify (Horikoshi and Tang 2015), to make figures for survival curves.

Though the experiment was not designed to quantify mortality rates among the treated bees, we report the numbers of treated individuals that were observed resuming sonication behavior (and then removed from the experiment), that were alive at the end of the experiment but not observed sonicating after treatment, and that were dead, in each of the treatment groups. We did not use statistical tests to assess differences among groups, because the experiment was not designed to test mortality, and we were unable to identify all of the bees at the end of the experiment.

We report un-adjusted p values, with no corrections to account for multiple comparisons. However, we discuss the possible tests that may be interpreted skeptically, due to relatively high p values.

Results

Sample sizes

After removing newly emerged queens and bees that were incorrectly marked or treated, we report 199 bees from four hives that were marked in our study—106 with bee tags and 93 with paint. We observed 105 of the marked bees resuming sonication behavior after being marked—34 with bee tags and 71 with paint. Of the 105 bees that received one of the imidacloprid or control treatments, 45 were observed resuming sonication behavior after being treated. The remaining 60 bees remained in the cages, but were not observed preforming sonication after being treated.

Wingbeat frequency, sonication frequency, and sonication length

We included only bees in the control and smallest dosage group (0 and 0.0515 ng imidacloprid, respectively) in this analysis, since we did not have large enough samples of the other two groups for analysis. Beyond the imidacloprid dose, no additional covariates improved the model, so we report wingbeat frequency, sonication frequency, and sonication length as dependent variables, with imidacloprid dose as the only independent variable. We found no evidence that wingbeat frequency, sonication frequency, or sonication length were affected by consuming 0.0515 ng of imidacloprid (MANOVA; Pillai test stat = 0.08; approx. $F_{(3,31)} = 0.9$; p value = 0.45).

Probability of resuming sonication

Figure 1 shows the cumulative percentage (inverse Kaplan–Meier curves) of bees that were observed sonicating on *S. lycopersicum* after consuming different doses of imidacloprid. For this analysis, we used bees that sonicated on both varieties of *S. lycopersicum*. When we reran this analysis, either including the tomato variety as a covariate or excluding bees that sonicated on *S. lycopersicum* "Sweet 100 Hybrid", we found no difference in the significance of coefficients associated with imidacloprid treatment. Our final Cox proportional hazard regression model included only imidacloprid treatment as a predictor variable ($\chi^2_{(3)} = 23.58$; p value < 0.00001). No other covariates made the model better. This model did not include individuals for which we could not record intertegular span, because we needed to have the same dataset in all models for likelihood ratio tests to be valid. However, we reran the final model with all individuals (including those without IT span measurements), and found no change in significant or non-significant coefficients.

Though the bees that ingested 0.0515 ng of imidacloprid did not show a marked difference in their probability of resuming sonication behavior ($\beta_{(0.0515\ ng)} = 0.44$; $z = 1.326$; p value = 0.18), the bees that ingested 0.515 ng of imidacloprid were marginally different than the control group ($\beta_{(0.515\ \ ng)} = -1.02$; $z = 1.98$; p value = 0.048)—this borderline p value would become non-significant if we adjusted for multiple comparisons, using the Bonferroni method. The bees that ingested 5.15 ng of imidacloprid showed markedly different behavior ($\beta_{(5.15ng)} = -2.73$; $z = 2.64$; p value = 0.0084).

Bee mortality

Figure 2 shows the number of bees that received each treatment, and their status at the end of the experiment. Bees that were observed sonicating after treatment were removed after observation, so they could not be classified as dead or alive at the end of the experiment. The sample sizes in Fig. 2 are different from the sample sizes used in the Cox regression, because we were unable to find some individuals at the end of the experiment, so we dropped them from the Cox regression.

Discussion

Based on previous research about the effects of pesticides on other aspects of pollen foraging (Feltham et al. 2014), we expected to see a significant difference in the buzz-pollination behavior of bumblebees that ingested imidacloprid. We did not find evidence that any of the mechanical aspects of behaviors we investigated—sonication frequency, sonication length, or wingbeat frequency—were different for bees that ingested 0.0515 versus 0 ng of imidacloprid. We were unable to analyze data for bees in groups that received higher doses of imidacloprid, because we rarely observed them performing buzz pollination after ingesting imidacloprid—this reflects that imidacloprid has sub-lethal or delayed lethal effects when consumed at doses above 0.5 ng per bee. However, in a separate study, we found that wingbeat frequency was not affected by doses of imidacloprid up to 1 ng per bee (unpublished data).

The probability of observing bees sonicating on *S. lycopersicum* after treatment was significantly lower for bees that consumed 0.515 or 5.15 ng of imidacloprid, relative to bees that consumed no imidacloprid (Fig. 1). We acknowledge that the p value for the 0.515 ng group was relatively high (p value = 0.048)—had we adjusted the p value to account for multiple comparisons, using the conservative Bonferroni method, this group would have a p value above the $\alpha = 0.05$ level. We also acknowledge that

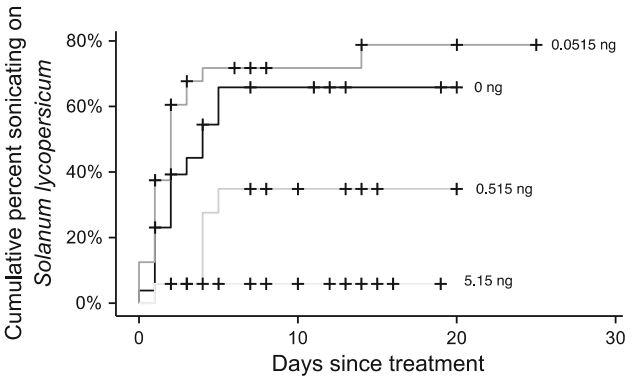

Fig. 1 Curves showing the cumulative percentage of bees that performed sonication on *Solanum lycopersicum* after ingesting different amounts of imidacloprid (n(0 ng) = 26, n(0.0515 ng) = 32, n(0.515 ng) = 17, n(5.15 ng) = 17). The *plus symbols* indicate censored data—bees that were never observed collecting pollen after treatment

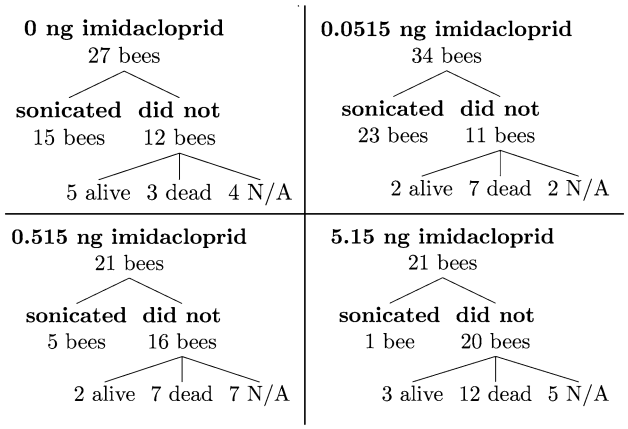

Fig. 2 The number of bees in each treatment group, and their status at the end of the experiment

our data were likely not completely independent for each individual—treating individuals with imidacloprid and removing foragers from the experiment (after they were observed sonicating again) likely influenced other bees within the hives, making them more likely to forage. Overall, though, it is clear that imidacloprid does affect the likelihood of bees continuing to engage in buzz pollination when ingested doses are above 0.5 ng bee^{-1}.

Though we were unable to analyze mortality in each of the treatment groups statistically, our data suggest a trend in mortality with increasing treatment doses (Fig. 2). We estimated mortality rates as follows: 11 % (3/27) for control, 21 % (7/34) for 0.0515 ng, 33 % (7/21) for 0.515 ng and 57 % (12/21) for 5.15 ng doses. The amounts of imidacloprid fed to bumblebees in this study were well below the LD_{50} values previously reported for bumblebees, which range from 20 to 40 ng per bee for 24–48 h after consumption (Decourtye et al. 2003; Marletto et al. 2003; Blacquiere et al. 2012).

The probable cause of death in our study was acute or delayed lethal effects of the pesticide, though other possible explanations exit. One hypothesis is that treated bees may not have been able to find the hive after leaving the nest to forage; studies on honeybees suggest that imidacloprid reduces navigation abilities (Bortolotti et al. 2003; Fischer et al. 2014), but a study on bumblebees suggests that navigation may not be the cause of impaired foraging (Feltham et al. 2014).

We saw no evidence that bees were able to clear imidacloprid from their systems after a period of time. If bumblebees in our study had cleared the imidacloprid from their bodies, we would have expected to see no difference in the pollination behavior of treated bees versus untreated bees, as post-treatment observations of pollination behavior did not begin until at least 24 h after treatment, and continued for up to several weeks. Alternatively, bees in our study may have in fact cleared imidacloprid from their bodies, but the effects of the treatment lasted well beyond the point when they had cleared the pesticide.

Our study had several limitations. First, treating bees with imidacloprid in a single dose does not represent the manner in which bees would be exposed in natural environments, but this method had the benefit of allowing us to precisely control the amount of pesticide ingested by bees. Second, though our study investigated only the effects of imidacloprid in nectar, pollen also can contain imidacloprid and its residues (Schmuck et al. 2001; Rogers and Kemp 2003; Stoner and Eitzer 2012). The *Solanum lycopersicum* plants in our study were never treated with neonicotinoid pesticides. However, the supplemental pollen feeder was filled with pollen collected from honeybees (http://www.koppert.com), and we cannot be sure that this pollen was free of neonicotinoid pesticides. However, we do not expect the pollen to significantly affect forager behavior for several reasons. First, foragers do not consume large amounts of pollen, relative to nectar intake, as pollen is primarily collected for the larvae. In honeybees, foragers eat only small amounts of pollen (Crailsheim et al. 1992). In bumblebees (*Bombus terrestris*), Malone et al. (2000) recorded workers eating 3.3–35.3 mg of pollen per day—a small amount, relative to the reported 125 to 215 µL of sugar syrup (50 % w/v) consumed per day. The concentration of neonicotinoid pesticides in pollen is often reported to be 8–20 ppb (Schmuck et al. 2001; Rogers and Kemp 2003; Bonmatin et al. 2003), though Mullin et al. (2010) found 206 ppb imidacloprid in honeybee pollen.

An interesting follow-up experiment would be to test bumblebees' ability to learn how to perform buzz pollination after consuming imidacloprid. Compared to nectar foraging, pollen collection is a more challenging behavior that has

been shown to require a substantial time to learn (Raine and Chittka 2007), and imidacloprid is known to hinder learning in bees (Han et al. 2010; Tan et al. 2015; Stanley et al. 2015a). In our experiment, bees were observed collecting pollen from *S. lycopersicum* at least two times before consuming imidacloprid (once prior to marking and again prior to imidacloprid treatment). Thus, bees had already learned how to process *S. lycopersicum* flowers before consuming imidacloprid. However, we might predict that bees who consume imidacloprid before performing buzz pollination would have a more difficult time learning to collect pollen by this method, or would have trouble collecting pollen as effectively as non-treated bees.

Conclusions

To sum up, our results show that imidacloprid in small doses can affect the buzz-pollination behavior of bumblebees. We did not find evidence that consuming 0.0515 ng imidacloprid affected bumblebees' wingbeat frequency, sonication frequency, or sonication length, and we were unable to test these variables in bees that consumed higher amounts of imidacloprid. However, we found that bumblebees who consumed 0.515 or 5.15 ng of imidacloprid were significantly less likely to resume sonication behavior after treatment, compared to bees subjected to a control (0 ng) treatment. In addition, we noted that many (but not all) of the bees that had consumed 5.15 ng of imidacloprid were dead at the end of our experiment, suggesting that future experiments are needed to test whether 5.15 ng imidacloprid—which is significantly below reported LD_{50} values for bumblebees—increases the mortality of foragers within a nest through either acute or delayed lethal effects.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical statement The manuscript has not been submitted to other journal simultaneously. It has not been published previously. The study has not been split into several parts to increase the quantity of submissions. The data have not been fabricated or manipulated. No theories or text are plagiarized, though the methods section is very similar to the methods presented in another submitted manuscript of ours. All authors have given consent to submit the article, and all authors have contributed significantly to the manuscript.

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